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How PEGylation Enhances the Stability and Potency of Insulin: A Molecular Dynamics Simulation

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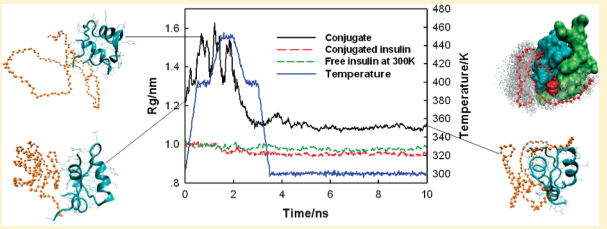
Supporting Information

ABSTRACT: While the effectiveness of PEGylation in enhancing the stability and potency of protein pharmaceuticals has been validated for years, the underlying mechanism remains poorly understood, particularly at the molecular level. A molecular dynamics simulation was developed using an annealing procedure that allowed an all-atom level examination of the interaction between PEG polymers of different chain lengths and a conjugated protein represented by insulin. It was shown that PEG became entangled around the protein surface through hydrophobic interaction and concurrently formed hydrogen bonds with the surrounding water molecules. In addition to enhancing its structural stability, as indicated by the root-mean-square difference (rmsd) and secondary structure analyses, conjugation increased the size of the protein drug while decreasing the solvent accessible surface area of the protein. All these thus led to prolonged circulation life despite kidney filtration, proteolysis, and immunogenic side effects, as experimentally demonstrated elsewhere. Moreover, the simulation results indicated that an optimal chain length exists that would maximize drug potency underpinned by the parameters mentioned above. The simulation provided molecular insight into the interaction between PEG and the conjugated protein at the all-atom level and offered a tool that would allow for the design of PEGylated protein pharmaceuticals for given applications.



Recent years have seen a proliferation of studies on therapeutic proteins and polypeptides, as driven by "omics" such as genomics, proteomics, etc., which are unveiling the molecular mechanisms of many diseases.[1] Marginal stability remains the major problem hindering both the production and utilization of protein therapeutics.[2,3] As for the administration of proteins, their potency is challenged by proteolysis, a relatively short serum half-life due to kidney filtration, and potential immunogenicity. Thus, much effort has constantly been directed toward finding stabilization methods to enhance the potency of protein therapeutics in vivo. To date, PEGylation (i.e., attaching PEG to a protein surface resulting in a PEG−protein conjugate), as first described by Abuchowski[4,5] in 1977, is one of the most effective and extensively applied methods for overcoming the deficiencies mentioned above.[6−8] Accordingly, a number of products have been introduced to the market: Adagen, Oncaspar, PEGIntron, Pegasys, Somavert, Neulasta, MIRCERA, and Cimzia.[9−11]

The progress mentioned above further fostered research into the interaction of the PEG polymer chain with the conjugated protein and its impact on the structure and activity of the protein. Basu et al.[12] reported an incremental improvement in the pharmacokinetics of interferon β-1b in response to an increase in the mass of the attached PEG. As for the PEGylation of interferon α-2,[13−15] G-CSF,[16,17] GRF,[18] and insulin,[19] whereas longer PEG chains reduced protein activity in vitro, they also produced prolonged in vivo circulation. Manjula and Acharya et al.[20,21] have shown that PEG chain length had no significant effect on the oxygen binding affinity of hemoglobin but increased the autoxidation rate as a function of PEG chain length upon conjugation of PEG onto Cys93(β). It has been reported that the enhancement of the conversion of nitrite to nitric oxide using PEGylated hemoglobin is independent of the number of attached PEG molecules.[22,23] López-Cruz et al.[24] have reported that the conjugation of laccase with PEG5000 appears to have marginal effects on the catalytic properties. Recently, Chiu et al.[25] observed an enhanced specific activity rate of 110% compared to that of the native counterpart for 2K, 5K, and 10K PEG-conjugated trypsin, but the 20K PEG-conjugated trypsin gave an activity lower than that of the native trypsin. Furthermore, the denaturation temperatures for trypsin and the PEGylated conjugate were nearly identical. Also for trypsin, Zhang[26] and Treetharnmathurot[27] have confirmed an improvement in the thermal stability of the PEG−trypsin conjugate in response to an increase in the PEG molecular weight from 350 to 5000, whereas Griebenow et al.[28,29] have shown that attaching more PEG units favors the thermal stability of α-chymotrypsin; a variation in the molecular weight of PEG from 700 to 5000, however, had no visible effect. In addition to PEG chain length, the PEGylation chemistry and conjugation sites can also influence the conformation of the PEG−protein conjugate; Acharya and co-workers have shown a "mushroom to brush" conformation transformation when the number of conjugation sites exceeds a critical number.[30−33] It may be concluded from these results that, whereas the effect of PEGylation in

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enhancing the stability and potency of protein pharmaceuticals is assured, our understanding of the interactions between PEG and the conjugated protein that underpin the stability and potency of PEGylated proteins remains far from adequate, particularly at the molecular level.

The rapid development of molecular simulation methods has allowed us to understand the molecular events underlying molecular functions and biochemical processes.[34−36] The challenge in applying molecular simulation techniques to the interactions between PEG and proteins is the intensive computing loading, a common problem for modeling biomacromolecules, particularly when all-atom scale information is pursued. Far fewer papers about simulating PEG−protein conjugates at the all-atom level are available.[20,37]

The objective of this study was to probe the interactions between PEG and a conjugated protein at the all-atom level. Here, insulin was chosen as a representative of protein therapeutics because of the relative abundance of experimental data,[19,38−43] which provided experimental fundamentals for both the modeling of the conjugate molecule and the subsequent simulation.

In this work, a PEG−insulin conjugate model was constructed with different PEG chain lengths and a simulated annealing procedure was developed to allow the system to achieve equilibrium at an accelerated speed without compromising the accuracy of the simulation. The simulation started with a molecular outlook of the configuration of PEG and conjugated insulin in an aqueous box. Then, the effects of PEGylation with polymers with different chain lengths on the potency and stability of insulin were examined and interpreted from the gyration radius, solvent accessible surface area, and secondary structure variations. The simulation results were compared with experimental observations reported in the literature. Molecular insights into PEGylation, as attempted in this study, are fundamentally important to the development of PEGylated protein therapeutics.

SIMULATION METHOD

The crystal structure of the insulin monomer was obtained from the Protein Data Bank (entry 4INS).[44] A PEG polymer model allowing different chain lengths was constructed and optimized with Gaussian03. Then PEG was linked to the insulin surface via an amide linkage at LysB29, as this is the unique lysine in insulin available for ε-NH_2 conjugation, represented in Figure 1. This is consistent with the conjugated site and PEGylated chemistry of Hinds et al.[19] GROMACS version 4.0.5[45,46] was used to perform MD simulations with the GROMOS96 43a1 force field.[47] The conjugate was solvated by explicit water using a simple point charge (SPC) model,[48] and ions were added to neutralize the system. Simulations were performed using general triclinic cell geometry and periodic boundary conditions. NVT ensemble and V-rescale thermostat[49] were used for temperature coupling with a coupling constant of 0.1 ps. A twin-range cutoff of 0.9/1.4 nm for van der Waals interactions was applied, and the particle mesh Ewald algorithm[50] was used for Coulomb interactions with a switching distance of 0.9 nm. Neighbor lists were utilized and updated every 10th integration step. The LINCS algorithm[51] was used to constrain all bonds. Molecular dynamics simulation was performed with a time step of 0.002 ps, on a personal computer cluster with 20 quad-core AMD Opteron 2379 HE processors (2.4 GHz).

During a run, the PEG−insulin molecule with different chain lengths or free insulin was placed in the center of a simulation box of sufficient size. The system containing the conjugate molecule and solvent was energy minimized to 1000 steps of steepest descent minimization and converged at a value lower than 200 kJ mol^{-1} nm^{-1} at 300 K. If the procedure failed, a combined conjugate gradient algorithm and a steepest descent algorithm were used, that is, performing one steepest descent step every 100 conjugate gradient energy steps, until the energy converged. A 1 ns position-restrained MD simulation was then performed by fixing the protein or PEG−protein conjugate coordinates and allowing the solvent molecules to equilibrate. Simulated annealing procedures were used for optimizing the conformation of PEG−insulin conjugates, and a leapfrog algorithm was used for integrating the Newtonian equations of motion. Taking a PEG−insulin conjugate with 10 polyethylene glycol units as an example, the procedure was performed by increasing the temperature from 300 to 400 K linearly within 0.5 ns and equilibrating at 400 K for 0.3 ns and then increasing the temperature to 450 K linearly in an additional 0.5 ns, equilibrating at 450 K for 0.2 ns, and then decreasing the temperature symmetrically and stabilizing at 300 K for an additional 7.2 ns. For the PEG−insulin conjugate with 10 PEG units, a 50 ns MD simulation was also performed at a constant temperature of 300 K to validate the simulated annealing procedure.

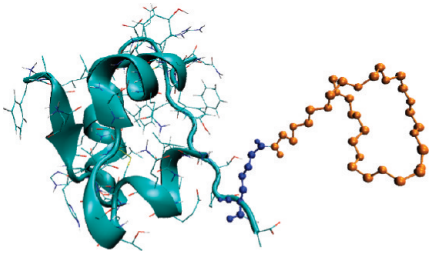

Figure 1. PEG−insulin conjugate model, in which insulin is shown as a cartoon. In this example, PEG is shown with 10 monomer units, represented by the CPK model (orange), and conjugated LysB29 is colored blue.

The thermostability was tested via "accelerated" experiments in silico. After the conformation of the conjugates with different PEG chain lengths had been optimized via simulated annealing molecular dynamics simulations, they were centered in the simulation box, the temperature was increased to 450 K with a V-rescale thermostat, and the system was kept running for 10 ns.

All analyses were performed on the basis of GROMACS version 4.5.1. The secondary structure of insulin was recognized via the DSSP algorithm,[52] and the snapshots were drawn with VMD version 1.8.6.[53]

RESULTS AND DISCUSSION

The Increased R_g and Reduced SASA of Insulin as a Result of PEGylation Indicated Enhanced Pharmacokinetic Parameters with Respect to Kidney Clearance and Proteolysis. To achieve the equilibrium state, we first optimized the PEG−insulin model using the molecular dynamics approach. For systems with higher PEG molecular weights, such as those having 50, 100, or 200 ethylene glycol units, each simulation run took hundreds of nanoseconds or even longer. Thus, we introduced the simulated annealing procedure to overcome computing constraints and allow the PEG−insulin conjugate to jump out of the local energy traps and proceed to the equilibrium state. The rmsd values of the PEG−insulin conjugate, conjugated insulin itself, and free insulin, which was used as a reference, are shown in Figure 2. To verify the simulated annealing procedure, a normal molecular dynamics

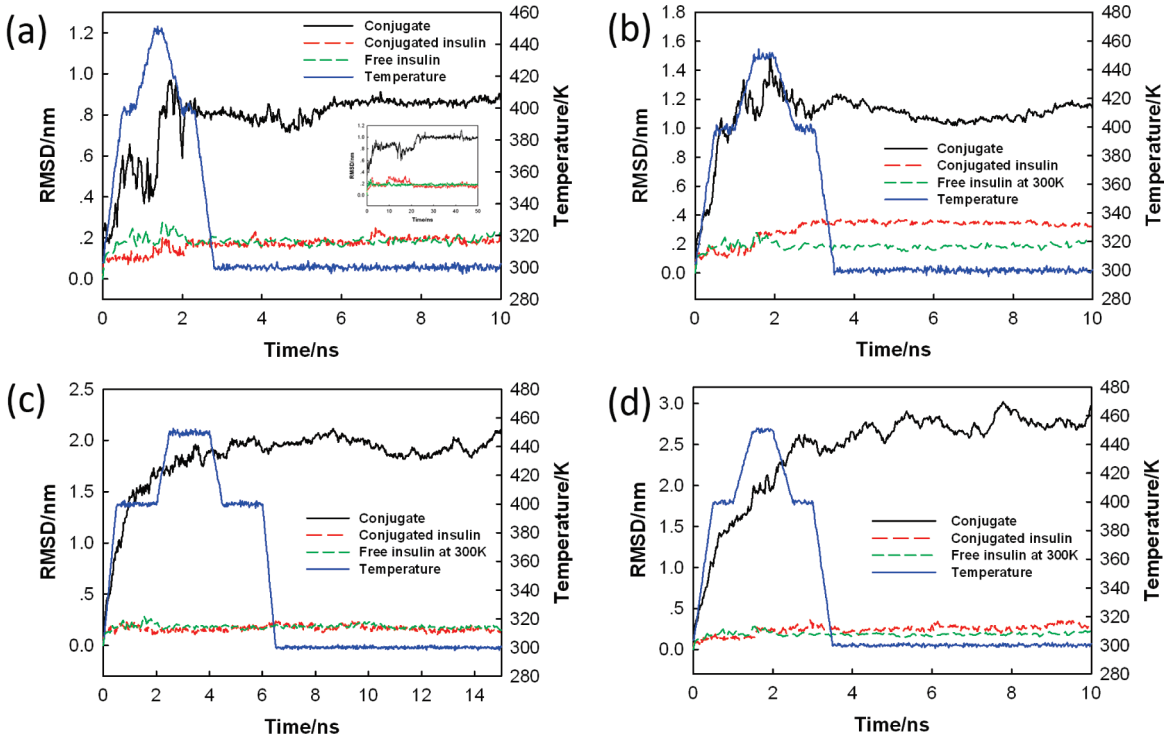

Figure 2. rmsd values of PEG−insulin conjugates and conjugated insulin with different PEG chain lengths under the simulated annealing procedure: (a) insulin−PEG10 conjugate (inset showing the system reached equilibrium in 50 ns with normal molecular dynamic simulation at a constant 300 K), (b) insulin−PEG50 conjugate, (c) insulin−PEG100 conjugate, and (d) insulin−PEG200 conjugate.

simulation of 50 ns and a molecular dynamics simulation with simulated annealing procedure of 10 ns were performed for the insulin−10 PEG conjugate. As shown in Figure 2a, the simulated annealing procedure arrives at the equilibrium state in 6 ns while the regular methods take ∼30 ns.

As seen in Figure 2, while the rmsd of the conjugate exhibits large fluctuations attributed to the movement of the flexible PEG chain, the rmsd of the conjugated insulin undergoes changes similar to those of free insulin with an average rmsd of 0.2 nm, as determined by X-ray diffraction.[44] The DSSP (Define Secondary Structure of Proteins) algorithm reveals the secondary structure of the conjugated insulin remains unchanged during simulation (Figure S1 of the Supporting Information). These outcomes suggest that attaching a PEG chain to the insulin surface does not affect the native conformation that underpins its biological activity. In other words, the PEG−insulin conjugate is able to reproduce the biological function of native insulin. The stabilization effect of PEGylated insulin has been confirmed by Hinds et al.[19] The circular dichroism spectroscopy and activity assay in vivo showed that PEG750−LysB29−insulin and PEG2000−LysB29−insulin conjugates had similar structural features and biological activity compared with those of free insulin, except that the PEG2000−LysB29−insulin conjugate had an attenuated activity (85% of that of free insulin). The activity loss of the larger PEG−insulin conjugate was likely attributed to the hindrance of PEG on the docking of insulin and its receptor.

Figure 3 shows the radius of gyration (R_g) and corresponding snapshots of conjugates with different PEG chain lengths. As the simulation proceeds, the R_g value of the conjugates decreases and stabilizes at a minimum value, representing the equilibrium state.

A simulated annealing method for searching for the optimal conformation was developed, as shown in Figures 2, with an increase in the temperature from 300 to 450 K in two steps. The energy barriers of the initial PEG conformation are surpassed, allowing the molecule to search for its equilibrium state once the temperature starts to decrease and reaches 300 K again. From the snapshots, one can see that, once the simulation is started, PEG in the collapsed state tends to be stretched at the beginning of the annealing but eventually forms a compact aggregate and interacts with insulin via hydrophobic interactions. The process of searching for the optimized conformation is affirmed by principal component analysis (PCA) in Figure S2 of the Supporting Information, similar to protein folding in solution, which is characterized by a "collapse−rearrange" sequence[54,55] and had little relationship with the initial conformation of the PEG chain (Figures S3 and S4 of the Supporting Information).

Figure 4 shows the statistical distribution of the polymer with 50 PEG units around an insulin molecule in the equilibrium state. Here, a distinctive polymer layer is formed to protect the conjugated insulin that remains in its native conformation; moreover, the PEG chain remains flexible in the equilibrium state. The entire conjugate, however, appears as a "mushroomlike" molecule. The assembly of the PEG chain at the protein surface may undergo significant changes. One example was reported by Acharya et al., who observed the transition of the conformation of the PEG−hemoglobin and PEG−albumin conjugates from mushroom to "brush".[31,33]

Figure 5 shows the SASAs of the conjugated insulin obtained with PEG with different chain lengths, sampling from the equilibrium stage. Compared to the SASA of free insulin, the attachment of a PEG chain reduces the solvent accessible area of both hydrophobic and hydrophilic residues. This is the "shielding effect" of PEG as an inert polymer. Moreover, the shielding effect is intensified when the size of the SASA probe increases from 0.14 to 0.5 nm. This elucidates how PEG prevents proteolysis and

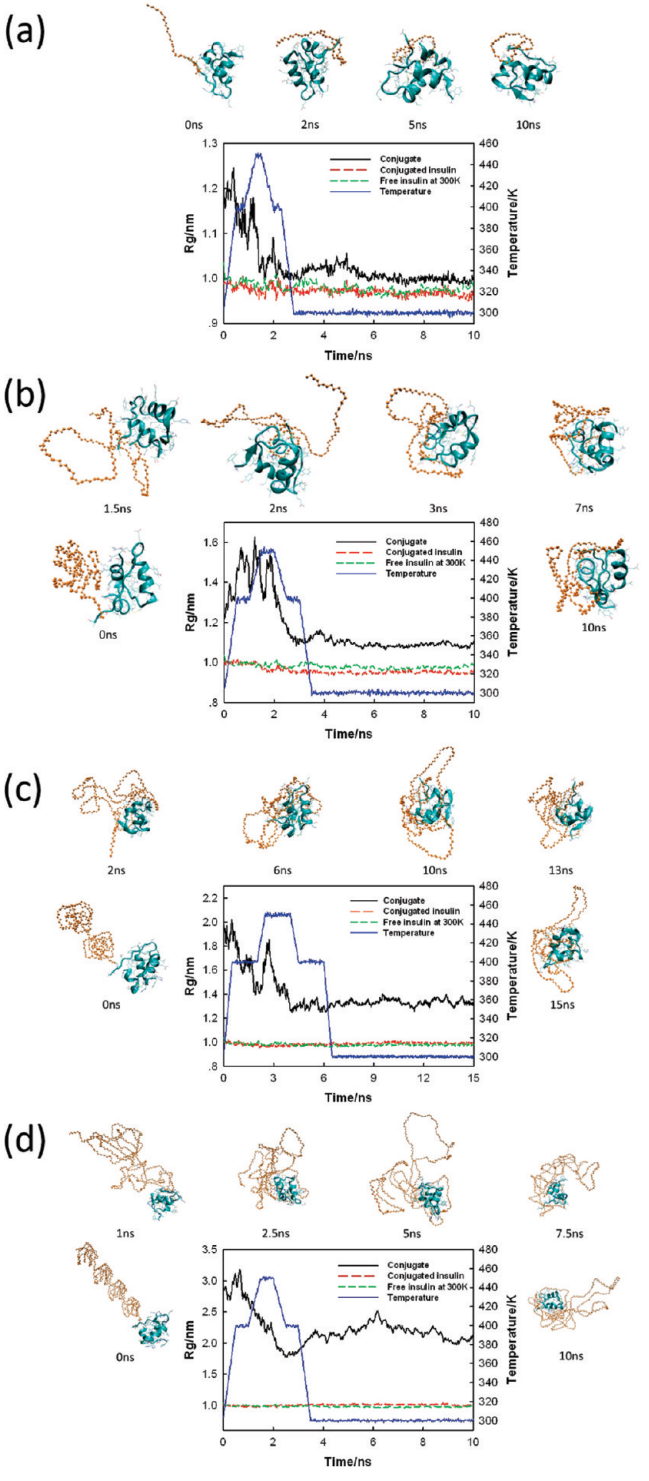

Figure 3. Radii of gyration (R_g) and corresponding snapshots of PEG−insulin conjugates with different chain lengths. Insulin is shown as a cyan cartoon, and PEG is shown as an orange CPK model: (a) insulin−PEG10 conjugate, (b) insulin−PEG50 conjugate, (c) insulin−PEG100 conjugate, (d) insulin−PEG200 conjugate.

blocks the receptors of immune cells, for the proteases and receptors are much bigger than the solvent molecules.

As shown in Figure 6, the protective effect is likely to have a linear relationship with the natural logarithm of PEG molecular

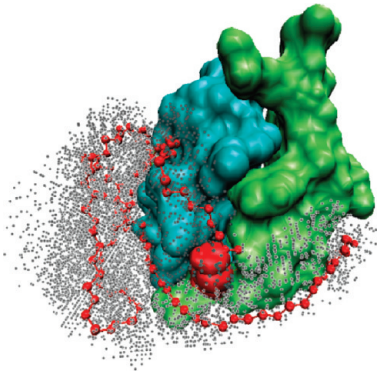

Figure 4. Distribution of PEG around an insulin molecule in an insulin−PEG50 conjugate during the last 4 ns of the molecular dynamic simulation. Insulin is shown as a surface model, with chain A colored cyan and chain B lime; the conjugated LysB29 residue is shown as a red VDW model, and a typical PEG chain is shown as a red CPK model.

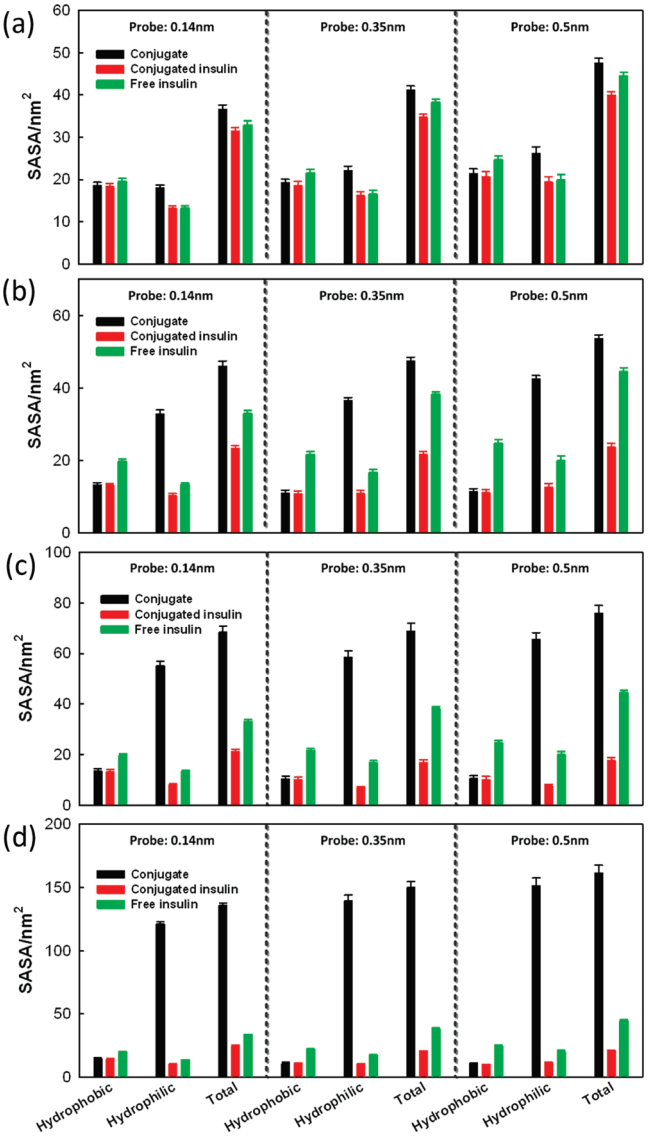

Figure 5. SASAs of PEG−insulin conjugates: (a) insulin−PEG10 conjugate, (b) insulin−PEG50 conjugate, (c) insulin−PEG100 conjugate, and (d) insulin−PEG200 conjugate.

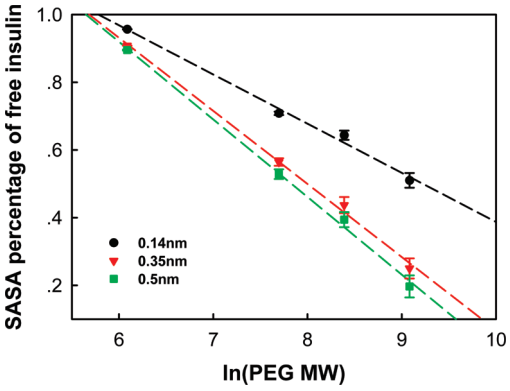

Figure 6. SASAs of conjugated insulin with different probe radii as a percentage of free insulin.

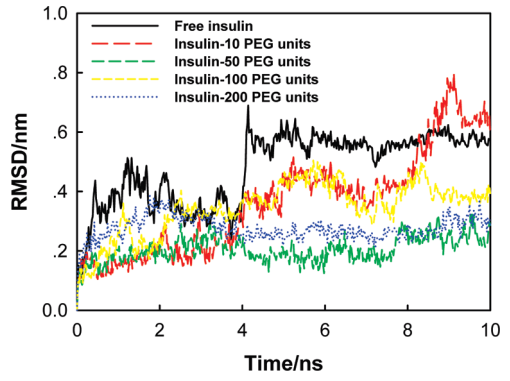

Figure 7. rmsds of free insulin and conjugated insulin at 450 K.

weight. In other words, a larger PEG molecule covers more surface area of the protein and thus is more effective in shielding it from enzyme degradation and reducing the immunogenicity of the protein. This conclusion had been validated by Hinds et al.[19] and the second-generation PEGylated protein pharmaceuticals,[7] such as PEG-uricase (Puricase)[56] and PEG-L-asparaginase (Oncaspar).[57,58] Moreover, the results shown in Figure 6 also indicate a limit of reduced SASA and a best molecular weight of PEG exist. In practice, the conjugation of larger PEG molecules onto insulin is hindered by the reduced accessibility of the reaction site and the extended reaction time required, both of which may lead to reductions in the conjugation efficiency and yield of protein activity.

rmsd and Secondary Structure Analyses Showing the Enhanced Stability of Conjugated Insulin. The stabilization effect of PEGylation on protein pharmaceuticals has been validated by numerous experiments,[19,24−29] and a similar conclusion can also be drawn for glycosylated protein.[59,60] Here, we tested the stability of free insulin and PEG−insulin conjugates at 450 K in silico. Such a high temperature may accelerate the denaturation of the protein. Figure 7 shows the rmsd fluctuations of native free insulin with respect to conjugated insulin. While the free insulin denatures quickly during the first 2 ns, its conjugated counterpart remains in the native conformation except when it is linked to PEG with 10 ethylene glycol units, in which case it undergoes significant changes at the end of the simulation. This latter effect may be attributed to the insufficient chain length of PEG that fails to provide the required magnitude of hydrophobic interaction with the conjugated insulin.

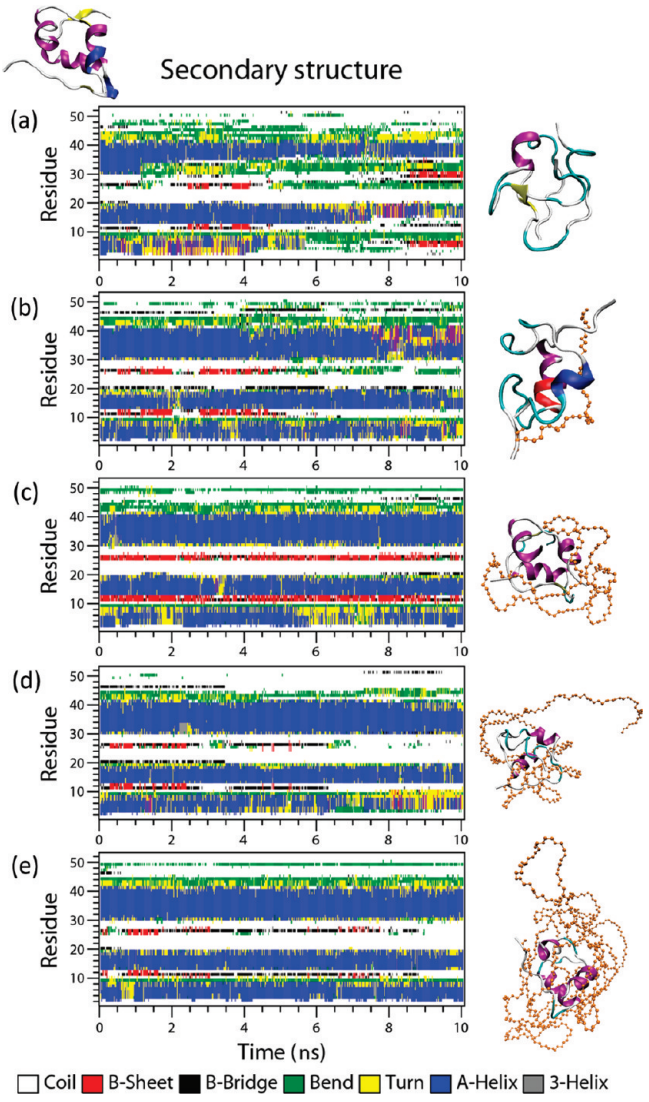

Figure 8. Secondary structure analyses of free insulin and conjugated insulin at 450 K. The snapshot at the top left shows the initial native insulin conformation, and the snapshots at the right show the final conformations of free insulin and conjugated insulin, where insulin is colored by its structure and PEG is represented as an orange CPK model: (a) native free insulin, (b) insulin−PEG10 conjugate, (c) insulin−PEG50 conjugate, (d) insulin−PEG100 conjugate, and (e) insulin−PEG200 conjugate.

Figure 8 shows the secondary structure of the insulin domain, which consists of three α-helices, including two helices in chain A and one in chain B, under the same conditions of accelerated denaturation in silico at 450 K; the final snapshots are also presented. Free insulin experiences extensive structural fluctuations and loses nearly all of its native structure. On the other hand, conjugated insulin retains its native secondary structure. Similar results of this structural transition were obtained by thermodynamic unfolding and H−D exchange FTIR analysis of PEGylated chymotrypsin, as reported by Griebenow et al.,[29] which showed the reduced structural dynamics of the conjugated protein.

In addition to thermal deactivation, random interactions with water molecules also lead to protein deactivation. The radial distribution function of water around the conjugated insulin has been calculated and is plotted in Figure 9.

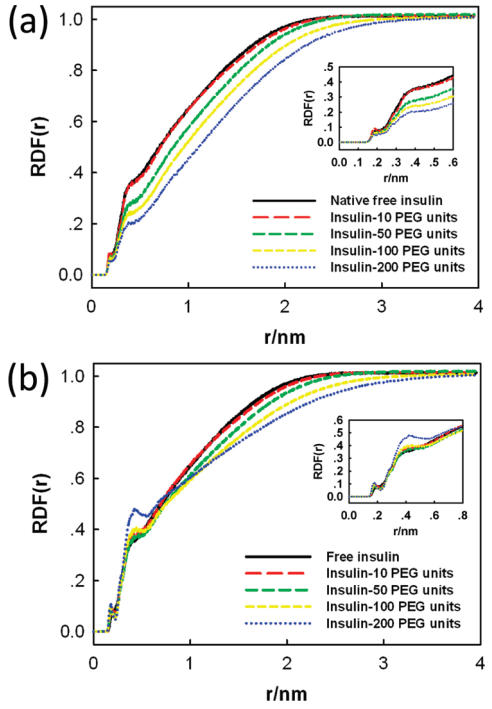

Figure 9. (a) Radial distribution function of water around free insulin and conjugated insulin. (b) Radial distribution function of water around free insulin and whole conjugates.

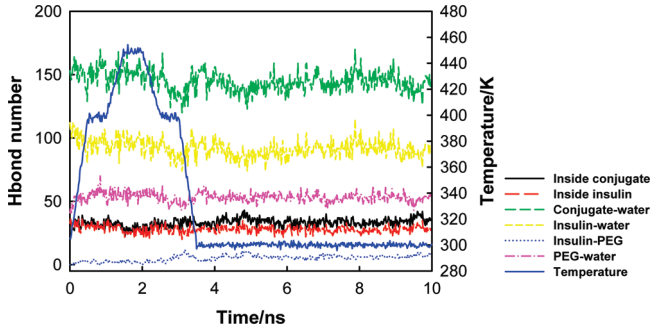

Figure 10. Analysis of the number of hydrogen bonds involving the insulin−PEG50 conjugate.

Figure 9a shows that the density of water around the insulin moiety decreases as the PEG chain length increases. In other words, the longer the PEG chain, the more water molecules are excluded from the surface of the insulin. The hydrophilicity of PEG−insulin conjugates is shown in Figure 9b. The distribution of water around the conjugates is similar to that around native free insulin, and there is even an enrichment of water density around the larger PEG moieties, which indicates that the affinity of PEG for water generates a water layer surrounding the PEG−insulin conjugate.

Energy Analysis of PEG−Insulin Conjugates. From studies of protein refolding, a conclusion that a lower free energy favors the improved stability of the molecule has been reached.[61,62] In other words, molecular energy is a key to understanding the effect of PEG chain length on the stability of the conjugated protein, as described in the introductory section. Thus, we performed hydrogen bond and energy analysis of PEG−insulin conjugates with different PEG chain lengths. The results are shown in Figures 10 and 11, taking the insulin−PEG50 conjugate as an example.

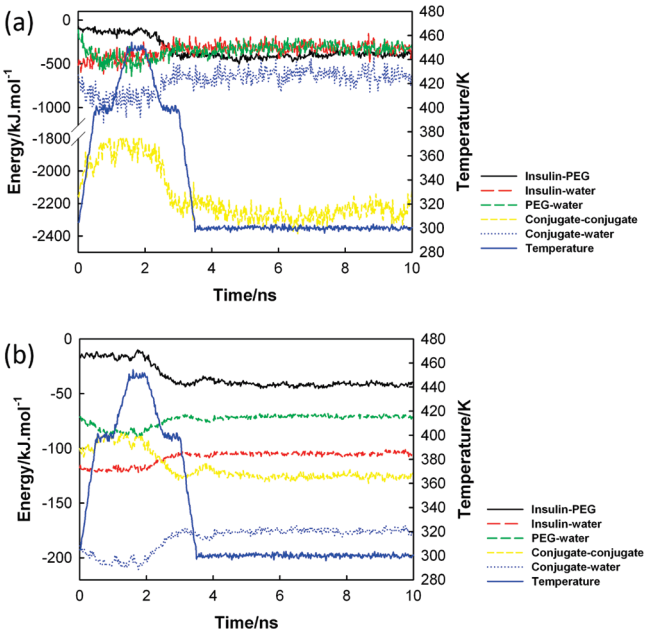

Figure 11. Lennard-Jones (LJ) energy analysis of the insulin−PEG50 conjugate: (a) the short-range component of the LJ energy and (b) the long-range component of the LJ energy.

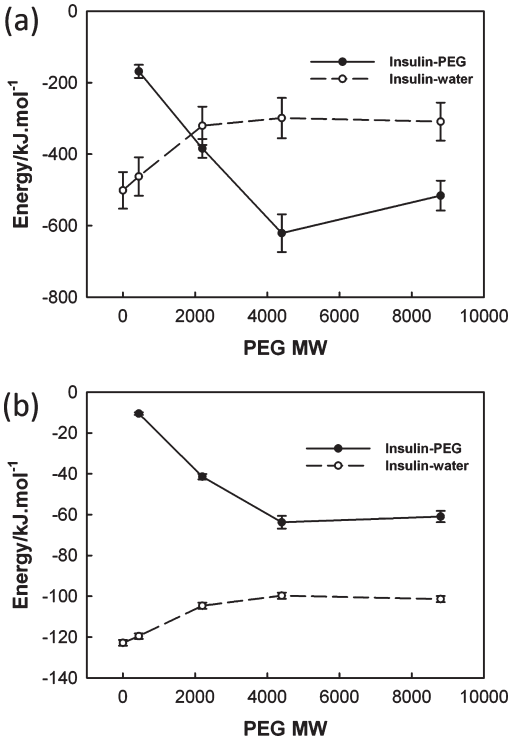

Figure 12. LJ energy comparison for PEG−insulin conjugates with different chain lengths: (a) the short-range component of the LJ energy and (b) the long-range component of the LJ energy.

(Results obtained using PEG with chain lengths of 10, 100, and 200 PEG units are presented in the Supporting Information.)

It can be seen from Figure 10 that the number of hydrogen bonds remains stable during the simulated annealing process, although the conjugate experiences more significant variations in rmsd values

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indicating the extension and collapse of the PEG chain, as shown in Figure 2.

A Lennard-Jones (LJ) energy analysis elucidates the interactions among conjugated insulin, PEG, and water during the simulated annealing process, and the results are shown in Figure 11. The LJ energy between insulin and PEG is close to zero initially because the initial conformation is built as a "linking" of insulin and PEG as two isolated moieties. The interaction between the two parts is strengthened as the simulated annealing proceeds and reaches a stable state with a short-range LJ component of −400 kJ/mol and a long-range LJ component of −40 kJ/mol. Panels a and b of Figure 11 also show the simultaneous intensification of the LJ interactions within the conjugate and weakening of the LJ interactions between water and the solute (conjugated insulin, PEG, and the whole conjugate). These results demonstrate that the main driving force of the entangled PEG structure around insulin in the PEG−insulin conjugate is hydrophobic interactions. This is partially supported by the prolonged retention times, relative to the parent proteins, when PEG−G-CSF[63] and PEG−hemoglobin[20] conjugates are detected using reversed phase high-performance liquid chromatography, corresponding to enhanced hydrophobicity. Meanwhile, PEG interacts with the surrounding water molecules through large numbers of hydrogen bonds.

As shown in Figure 12, both the long-range and short-range components of LJ energy involving insulin and PEG increase with respect to increases in PEG molecular weight. This suggests that the intermolecular hydrophobic interactions of the conjugate are strengthened in response to an increase in PEG chain length. Accordingly, the long- and short-range components of LJ energy involving insulin and water decrease with an increase in PEG molecular weight. This suggests an enhanced shielding effect of PEG, which simultaneously replaces the LJ interactions between insulin and water. Further increases in PEG chain length over 100 PEG units, however, result in marginal enhancement of the shielding effect. This indicates there is a particular PEG mass that optimizes insulin potency and stability.

CONCLUSIONS

This study examined the interactions between PEG and a conjugated protein represented by insulin, with emphasis on the effects of changes in the chain length of PEG on the stability, proteolysis, and immunogenicity that underpin the performance of insulin in vivo. The MD simulation showed that conjugation with PEG directly resulted in a large molecular volume of the conjugate and a less accessible surface area of the insulin moiety. The reduction in SASA has a linear relationship with the natural logarithm of PEG molecular weight, which indicates a best chain length exists for the PEG−protein conjugate.

We tested the stability of our conjugate model under conditions of accelerated denaturation. Conjugates demonstrated excellent thermal stability and maintained their secondary structures at 450 K, while free insulin lost its native conformation completely. Conjugation with PEG with 10 ethylene glycol units, however, could not provide an adequate magnitude of hydrophobic interactions with insulin and thus gave a marginal stabilization effect.

Furthermore, we explored the mechanism and driving force underpinning the stabilization of conjugated insulin. Hydrogen bond and LJ energy analyses showed the driving force for the entangled structure of PEG is hydrophobic interaction rather than hydrogen bonding. PEG plays a dual role in the conjugate: on the one hand, PEG interacts with insulin via hydrophobic interaction, and on the other hand, PEG establishes large numbers of hydrogen bonds with water. The special character of PEG enhances the stability of the conjugated protein and maintains an aqueous layer that retains the water molecules necessary for the biological function of the protein. The simulation indicates that an optimal chain length exists that will maximize potency, underpinned by the parameters described above. As for molecular dynamics simulation, the simulation time, space, force field, and number of sampling are the major factors determining the accuracy of the simulated results. Nevertheless, the simulation, as also attempted by this study, provides molecular insight into the PEG−protein conjugate, which is essential to the design, fabrication, and application of PEGylated protein.

ASSOCIATED CONTENT

Supporting Information. Details of analysis tools in the molecular dynamics simulation, principal component analyses of the first two eigenvectors in the simulating trajectories with different stages, and additional structural and energy analyses of PEG−insulin conjugates. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS

DSSP, defined secondary structure of proteins; G-CSF, granulocyte colony-stimulating factor; GRF, growth hormone-releasing hormone; LJ, Lennard-Jones; MD, molecular dynamics; PEG, polyethylene glycol; R_g, radius of gyration; rmsd, root-mean-square difference; RDF, radial distribution function; SASA, solvent accessible surface area; PCA, principal component analysis.

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